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Environmental Study of Azole-Resistant *Aspergillus fumigatus* and Other Aspergilli in Austria, Denmark, and Spain[▽]

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A single mechanism of azole resistance was shown to predominate in clinical and environmental *Aspergillus fumigatus* isolates from the Netherlands, and a link to the use of azoles in the environment was suggested. To explore the prevalence of azole-resistant *A. fumigatus* and other aspergilli in the environment in other European countries, we collected samples from the surroundings of hospitals in Copenhagen, Innsbruck, and Madrid, flowerbeds in an amusement park in Copenhagen, and compost bags purchased in Austria, Denmark, and Spain and screened for azole resistance using multidish agars with itraconazole, voriconazole, and posaconazole. EUCAST method E.DEF 9.1 was used to confirm azole resistance. The promoter and entire coding sequence of the *cyp51A* gene were sequenced to identify azole-resistant *A. fumigatus* isolates. *A. fumigatus* was recovered in 144 out of 185 samples (77.8%). Four *A. fumigatus* isolates from four Danish soil samples displayed elevated azole MICs (8%), and all harbored the same TR/L98H mutation of *cyp51A*. One *A. lentulus* isolate with voriconazole MIC of 4 mg/liter was detected in Spain. No azole-resistant aspergilli were detected in compost. Finally, *A. terreus* was present in seven samples from Austria. Multi-azole-resistant *A. fumigatus* is present in the environment in Denmark. The resistance mechanism is identical to that of environmental isolates in the Netherlands. No link to commercial compost could be detected. In Spain and Austria, only *Aspergillus* species with intrinsic resistance to either azoles or amphotericin B were found.

The saprophytic molds of the genus *Aspergillus* are found worldwide. They are present in soil and decaying organic matter, and most of the species have a high sporulation capacity (4, 17). The conidia can be dispersed to the ambient air and inhaled and eventually cause infection in a susceptible host. People with compromised immune systems and damaged lung architecture are among the main risk groups (24). Azoles, such as itraconazole, voriconazole, and posaconazole, are among the recommended first-line drugs in the treatment and prophylaxis of aspergillosis (36). Amphotericin B and the echinocandins (caspofungin, micafungin, and anidulafungin) are other drugs with clinical activity against *Aspergillus* spp. (36). *Aspergillus fumigatus* is the *Aspergillus* species responsible for the majority of invasive infections and chronic pulmonary cases of aspergillosis (7, 36). In contrast to *A. terreus*, which is intrinsically resistant to amphotericin B, and certain recently described species such as *A. lentulus* and *A. pseudofischeri*, which are intrinsically resistant to azoles (1), *A. fumigatus* is normally susceptible to all three antifungal drug classes. However, in recent years, a rising proportion of patients with aspergillosis caused by *A. fumigatus* isolates with acquired azole resistance have been reported (33).

The most common mechanism of azole resistance is an alteration of the target of the azole drugs. The target protein, the lanosterol 14-α-demethylase, is a key enzyme in the biosynthetic pathway of ergosterol, the predominant sterol in the cell membranes of most fungi. Mutations in the *cyp51A* gene, which encodes this target, have been shown to cause resistance (5, 8, 18, 20, 22).

Two patterns of azole resistance have emerged in *A. fumigatus*. First, the fungus may become resistant through exposure to azoles in the patient. This pattern was observed in cases of aspergilloma and chronic pulmonary aspergillosis in the United Kingdom (12). Eighteen different amino acid substitutions were detected in the *cyp51A* gene (12). Second, azole resistance may develop in the environment through the exposure of the fungus to azole fungicides used in agriculture and in material preservation. This pattern has been suggested in the Netherlands (35). The hypothesis was supported by the finding that a single resistance mechanism, a substitution at codon 98 of the *cyp51A* gene combined with a tandem repeat of 34 bp in the promoter region (TR/L98H), was found in 94% of the resistant isolates (30). Furthermore, this resistance mechanism was recovered both in azole-naïve patients (34) and in environmental samples (soil, compost, seeds, air, and water) (29). The consequence of this type of resistance development is that patients at risk can be exposed to and infected by azole-resistant strains in the environment.

Here, we report for the first time the detection of azole-resistant *A. fumigatus* with the TR/L98H resistance mechanism in Denmark in an environmental survey of the surroundings of main hospitals and compost conducted in Innsbruck, Austria (A); Copenhagen, Denmark (DK); and Madrid, Spain (ES).

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TABLE 1. Frequency of *Aspergillus* spp. in environmental samples from Denmark, Austria, and Spain

Sample source	No. of samples	Samples that grew:					
		A. fumigatus		*A. terreus*		Other aspergilli	
		No.	%	No.	%	No.	%
Hospitals							
Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark	27	17[a]	63.0	0	0	7[b]	25.9
University Hospital Innsbruck, Innsbruck, Austria	25	25	100.0	1	4.0	4[c]	16.0
Hospital Clinico San Carlos and Hospital Niño Jesus, Madrid, Spain	31[d]	17	54.8	0	0	30[e]	96.8
Park							
Tivoli Gardens, Copenhagen, Denmark	23	21[f]	91.3	0	0	2[c]	8.7
Compost							
Denmark	26	17	65.4	0	0	0	0
Austria	25	24	96.0	6	24.0	1[c]	4.0
Spain	28	23	82.1	0	0	7[c]	25.0

[a] One of 17 was azole resistant by the EUCAST method and had the TR/L98H mutation of Cyp51A.
[b] *A. niger*, 5; *A. flavus*, 1; *A. nidulans*, 1.
[c] *A. niger*.
[d] Fifteen samples (including three indoor flowerpot samples) from Hospital Clinico San Carlos and 16 samples from Hospital Niño Jesus.
[e] *A. niger*, 22; *A. nidulans*, 3; *A. flavus*, 2; *A. lentulus*, 1; *Aspergillus* sp., 2.
[f] Three of 21 were azole resistant by the EUCAST method, and all three had the TR/L98H mutation of Cyp51A.

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MATERIALS AND METHODS

Collection of samples. Soil was collected from flowerbeds surrounding the tertiary care university hospitals in Copenhagen (Rigshospitalet [~1,100 beds]) (27 samples), Innsbruck (Innsbruck University Hospital [~1,500 beds]) (25 samples), and Madrid (Hospital Clinico San Carlos [~1,200 beds] and Hospital Niño Jesus [~35 pediatric oncology beds]) (15 and 16 samples, respectively); from flowerbeds in an amusement park in the center of Copenhagen (Tivoli Gardens) (23 samples); and from compost bags purchased in Denmark (26 samples), Austria (25 samples), and Spain (28 samples). Environmental sampling was carried out in June, July, and August 2009.

Plating of soil samples and identification. The samples were handled and plated according to previously described methods (29) with minor modifications. Two grams of each sample was suspended in 5 ml 0.2 M NaCl with 1% Tween 20 and vortexed. Subsequently, 100 μl of this suspension was plated on Sabouraud dextrose agar (SAB) (DK, SAB pH 4 [Statens Serum Institut, Diagnostika, Hillerød, Denmark]; ES and A, SAB supplemented with chloramphenicol [Oxoid, Madrid, Spain, and Merck, Vienna, Austria]) and 50 μl in each of the wells of a four-well multidish plate containing RPMI 1640–2% glucose agar (AMT) supplemented with itraconazole (4 mg/liter), voriconazole (1 mg/liter), and posaconazole (0.5 mg/liter), and no antifungal (positive-control well) (Balis Laboratorium V.O.F., Boven Leeuwen, Netherlands) (31). Plates were incubated at 37°C and examined for growth at 24, 48, and 72 h.

Aspergillus colonies were identified according to microscopic and macroscopic morphologies (6). The interpretation of the plates was as follows. If pure a culture of an *Aspergillus* sp. was observed in SAB and no growth in azole wells of the AMT agar, the isolate was categorized as an azole-susceptible *Aspergillus* sp. If an *Aspergillus* sp. was combined with other filamentous fungi, the *Aspergillus* sp. was isolated to obtain a pure culture and inoculated with a swab on AMT to check for azole resistance. Finally, if an *Aspergillus* sp. was not detected on SAB or AMT, no *Aspergillus* was present in the sample. Samples with azole-resistant *A. fumigatus* were retested by testing another 2 grams of soil from the sample.

A. fumigatus isolates which were resistant to one or several azoles were incubated at 48°C to separate cryptic *A. fumigatus* complex isolates from *A. fumigatus* sensu stricto.

Aspergillus isolates which could not be identified to the species level by traditional methods or by incubation at 48°C were identified by amplification of a fraction of the β-tubulin gene using the primer set Tub5 (5′-TGACCCAGCAG ATGTT-3′) and Tub6 (5′-GTTGTTGGGAATCCACTC-3′) as previously described (21).

Susceptibility testing and breakpoints. *Aspergillus* isolates which grew on azole-containing agar were *in vitro* susceptibility tested according to the EUCAST microdilution method (28). The endpoints were recorded at 48 h and defined as the antifungal concentrations that produced a complete inhibition of visual growth. The antifungal agents used were itraconazole (range [ES/DK], 0.015/0.03 to 8/4 mg/liter) (DK, Sigma-Aldrich, Brøndby, Denmark; ES, Janssen S.A., Madrid, Spain), voriconazole (range, [ES/DK], 0.015/0.03 to 8/4 mg/liter) (DK, Pfizer, Ballerup, Denmark; ES: Pfizer S.A., Madrid, Spain), and posaconazole (range, [ES/DK], 0.015/0.03 to 8/4 mg/liter) (DK, Schering-Plough, Glostrup, Denmark; ES, Schering-Plough, Kenilworth, NJ). We considered *A. fumigatus* susceptible when the MICs for itraconazole and voriconazole were ≤1 mg/liter and that for posaconazole was ≤0.25 mg/liter (26, 27, 33).

PCR amplification and sequence analysis of *cyp51A* gene. Conidia from each strain (R13, T11, T18, T22, and *A. fumigatus* wild-type strain CM-237) were inoculated in 3 ml GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and grown overnight at 37°C. Mycelial mats were recovered and subjected to DNA extraction (19). The promoter and full coding sequence of *cyp51A* were amplified by PCR, and both strands were sequenced as described previously (8). The sequences were compared to the sequence of an azole-susceptible wild-type *A. fumigatus* isolate (CM-237).

Use of azoles in agriculture and human medicine. In Denmark, annual reports on the amounts of fungicides sold (kg) and of antifungals in human medicine (defined daily dosages [DDD]) are published by the Danish Environmental Protection Agency and the Danish Medicines Agency (http://www.medstat.dk/PackStatDataViewer.php), respectively. Conversion of DDD to kg was performed using WHO conversion tables (http://www.whocc.no/atc_ddd_index/). In Austria and Spain, the corresponding environmental agencies were contacted; however, no data regarding fungicide use were available.

RESULTS

A total of 185 environmental samples were collected (106 soil and 79 compost samples). The identification of *Aspergillus* spp. by type of sample and country is shown in Table 1. *A. fumigatus* was present in 77.8% of the samples and was isolated in samples from all three countries. The detection rate ranged from 54.8% (in samples from hospital surroundings in Madrid) to 100.0% (in samples from hospital surroundings in Innsbruck). *A. terreus* was detected in seven samples from Austria.

TABLE 2. Results of primary plating, EUCAST susceptibility testing, and Cyp51A amino acid substitution and *cyp51A* gene promoter alteration determination for four azole-resistant *A. fumigatus* isolates

Sample isolate	Growth of *A. fumigatus* by primary plating on azole agar[a]				MIC (mg/liter; Madrid/Copenhagen) determined by EUCAST			Cyp51A alterations
	ITC (4 mg/liter)	VRC (1 mg/liter)	POS (0.5 mg/liter)	Control (no antifungal)	ITC	VRC	POS	
R13	−	+	−	+	>8/>4	4/4	0.5/1	TR/L98H[b]
T11	+	−	+	+	>8/>4	4/4	0.5/2	TR/L98H
T18	+	−	−	+	>8/>4	4/4	0.5/0.5	TR/L98H
T22	+	−	−	+	>8/>4	4/2	0.5/0.25	TR/L98H
CM-237[d]	−	−	−	+	0.25/ND[c]	0.5/ND	0.06/ND	None (wild type)

[a] Subcultured isolates from all four sites were able to grow in all three azole-containing wells. ITC, itraconazole; VRC, voriconazole; POS, posaconazole.
[b] TR/L98H, tandem repeat of 34 bp in promoter region and substitution of leucine with histidine at codon 98.
[c] ND, not determined.
[d] CM-237, azole-susceptible *A. fumigatus* strain.

Other aspergilli were also detected; the majority were from hospitals in Madrid and were predominantly *A. niger* (41/51, 80.4%) (Table 1).

Four *A. fumigatus* isolates grew in at least one of the azole-containing wells of the AMT agar (Table 2). Susceptibility testing confirmed elevated MICs for itraconazole (>4 mg/liter), voriconazole (2 to 4 mg/liter), and posaconazole (0.25 to 2 mg/liter). All four *A. fumigatus* isolates were able to grow at 48°C, thus excluding cryptic species of the *A. fumigatus* complex, and they were all isolated from sample sites in Copenhagen (one from the hospital surroundings and three from the park). Additionally, one *A. niger* isolate, one *A. nidulans* isolate, and one *A. lentulus* isolate from three samples were able to grow in at least one of the azole-containing wells of the AMT agar. The *A. niger* and *A. nidulans* isolates were both detected in separate samples from the surroundings of Copenhagen University Hospital, and the *A. lentulus* isolate was detected in a sample from one of the hospital sites in Madrid and grew in the voriconazole well of the AMT agar. For the *A. niger*, *A. nidulans*, and *A. lentulus* isolates, the itraconazole, voriconazole, and posaconazole MICs were 2, 0.5, and 0.5 mg/liter; 1, 4, and 0.5 mg/liter; and 0.5, 4, and 0.25 mg/liter, respectively.

Sequence analysis of the *cyp51A* gene showed the same T364A point mutation in all four azole-resistant *A. fumigatus* isolates, resulting in an amino acid change of leucine to histidine at codon position 98. This was combined with a 34-base-pair sequence that was duplicated in the promoter region (TR/L98H) (Table 2).

Analysis of Danish data from 2008 on the amounts of azoles sold for use in agriculture and material preservation and of medical systemic mold-active azoles showed that the former was 1,500 times higher than the latter (144,000 kg versus 94 kg; data are from the Danish Environmental Protection Agency and the Danish Medicines Agency).

DISCUSSION

For the first time, we show that azole-resistant *A. fumigatus* isolates can be recovered in environmental samples in Denmark and that the phenotype and resistance mechanism are identical to those that predominate in the Netherlands (TR/L98H). In fact, azole-resistant *A. fumigatus* of this type was detected in 8% (4/50) of the Danish soil samples and in 11% (4/38) of the Danish soil samples containing *A. fumigatus*. We also found that *A. terreus* was present exclusively in samples from Austria, which is in line with the high proportion of cases of invasive aspergillosis caused by this species in Innsbruck (16).

In addition, three *Aspergillus* isolates (one each of *A. niger*, *A. nidulans*, and *A. lentulus*) with itraconazole or voriconazole MICs of above 1 mg/liter were detected. The MICs were, however, either within the wild-type azole MIC ranges for these species or one step elevated, as with voriconazole and *A. nidulans* (MIC of 4 mg/liter) (1, 3, 9). Thus, the findings most likely reflect intrinsic reduced susceptibility compared to *A. fumigatus* rather than acquired resistance mechanisms.

The detection of azole resistance among *Aspergillus* spp. was based on the use of an azole-containing medium. Screening of environmental samples for the presence of azole-resistant *A. fumigatus* using azole-containing agar was first described using itraconazole at a concentration of 4 mg/liter (29). Since then, a susceptibility breakpoint of 1 mg/liter has been proposed (25, 33), which may lead to the question of whether resistant isolates may go undetected. However, so far the itraconazole MICs of the vast majority of isolates with resistance mechanisms associated with itraconazole resistance have been 8 mg/liter or greater (12, 33). Furthermore, in this study we included voriconazole (1 mg/liter) and posaconazole (0.5 mg/liter) agars. This method has been evaluated using a collection of 70 clinical *A. fumigatus* isolates, of which 50% had MICs above the suggested breakpoints for one or several azoles, demonstrating high sensitivity and specificity values of 94% and 99%, respectively (31). Thus, it is unlikely that a significant number of resistant isolates would not have been captured.

Hypothetically, the method used may result in the laboratory generation of resistance in the recovered *Aspergillus* isolates through the exposure to azoles. However, we believe that this *de novo* azole resistance generation is unlikely due to the short length of exposure (72 h of incubation) and the detection of dual alterations in the *cyp51A* gene, both of which are necessary to cause resistance (21). Furthermore, the samples from which azole-resistant *A. fumigatus* was detected were retested, which confirmed the resistant nature of the isolates. Nevertheless, a way to bypass this theoretical step for generation of azole resistance would be to use molecular tools for the direct detection of the resistance mechanism. To our knowledge, this has only been published for human specimens (32) and has not been applied to environmental samples.

Acquired azole resistance develops in fungi in response to azole exposure. This has been shown to occur in patients previously exposed to azoles (5, 14) and in the plant pathogenic fungi (35). Long duration of drug exposure and high numbers of reproducing fungi are important factors in the selection of resistance (2). The conditions are met in patients with cavitary *Aspergillus* disease and, indeed, in the soil, where azole compounds can persist for more than a year (11).

Azole fungicides, or demethylation inhibitors, are used commonly for plant protection by the European Union member states, and manufacturers recommend the use of 100 g/ha in the field (11). It is estimated that slightly less than half of the total acreage under cereal and grapevine production is treated annually with azole fungicides (10). In comparison, less than 5% of the total crop area is treated annually in the United States (10). In the Netherlands the use of azole fungicides has almost doubled since the mid-1990s (33), and since 1999 a similar trend has been observed in Denmark (data are from the Danish Environmental Protection Agency). Apart from being selected due to local use of azole fungicides, azole-resistant *A. fumigatus* might be introduced in the environment via the use of imported commercial compost, an ecological niche which has been suggested as a key component in resistance development (35). Indeed, in our study all sites with resistant *A. fumigatus* were flowerbeds with cultivated soil, and about half of the tested compost bags in Denmark and Austria originated from a country other than that where they were purchased. However, we did not detect any azole-resistant isolates in samples taken directly from compost bags, which may suggest that the emergence of azole resistance in Denmark is due to local use of azole fungicides rather than imported compost.

Questions still remain about the emergence and predominance of *A. fumigatus* isolates with the TR/L98H resistance mechanism in the environment. The azole fungicides have been used for decades, but only since 2000 has a high proportion of resistant isolates been observed (35). It has been suggested that selected agents among the newer agricultural azoles may be more prone to induce resistance and that the TR/L98H isolates may have an advantage with respect to fitness compared to other isolates with mutations (35). Azole-resistant *A. fumigatus* has previously been detected in the environment (23), including resistant isolates where no *cyp51A* mutations were identified (29). In our study we detected resistant *A. fumigatus* isolates only with the specific TR/L98H mutation in the *cyp51A* gene. However, due to the sample size we cannot exclude that other resistance mechanisms are present among environmental isolates in the countries participating in this study. Furthermore, to our knowledge, no azole-resistant *A. fumigatus* isolate from the environment has been shown to have some of the other mutations in the *cyp51A* gene, such as G54 and M220, which have been detected in clinical samples. This could be due to the overall limited number of environmental samples investigated so far or, in theory, because the screening agars are more efficient in detecting *A. fumigatus* isolates with the TR/L98H *cyp51A* mutation. However, the previously characterized azole-resistant *A. fumigatus* isolates harboring other types of *cyp51A* mutations also show itraconazole MICs of greater than 8 mg/liter (12), and thus growth in the itraconazole well of the screening agar would be expected.

A. fumigatus isolates with the TR/L98H resistance mechanism has been isolated from patients in Spain, France, Norway, United Kingdom, the Netherlands, and Belgium (12, 13, 15, 21, 30). In Denmark, azole-resistant *A. fumigatus* with the M220K resistance mechanism has been recovered from a cystic fibrosis patient previously exposed to azoles (12). Additional azole-resistant isolates have been detected but have not yet been subjected to sequence analysis of the *cyp51A* region (M. C. Arendrup and K. L. Mortensen, unpublished data). Management of aspergillosis is difficult due to negative cultures being commonplace, and many routine laboratories do not perform susceptibility tests on *Aspergillus* isolates. Thus, the prevalence of azole-resistant cases may be underdiagnosed, with a potential risk of inappropriate therapy. We believe that the findings in our study and the emerging reports on azole resistance suggest that susceptibility testing of clinically important *Aspergillus* isolates should be routinely performed. A high frequency of resistance in a population is of concern and, if confirmed in forthcoming investigations, may suggest that the possibility of azole resistance should be taken into account in primary patient care.

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